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Short title: *ptm* is not a *gun* mutant

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Seedlings lacking the PTM protein do not show a *genomes uncoupled (gun)* mutant phenotype[1]

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Summary sentence: The *ptm* mutant of Arabidopsis does not show a *genomes uncoupled* mutant phenotype and PTM is therefore unlikely to function in chloroplast-to-nucleus signalling as previously reported.

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Chloroplast development requires communication between the nucleus and the developing chloroplast to ensure that this process is optimised (Jarvis and López-Juez, 2013; Chan et al., 2016). This is especially true during de-etiolation as mis-regulation of chloroplast development can lead to seedling death from photo-oxidative damage. Retrograde signalling from the developing chloroplast (plastid) to the nucleus, which is termed biogenic signalling (Pogson et al., 2008), can be revealed using either the bleaching herbicide Norflurazon (NF), an inhibitor of carotenoid synthesis, or the plastid translation inhibitor, lincomycin (Lin) to damage the plastid. Under these conditions there is a strong down regulation of hundreds of nuclear genes (Koussevitzky et al., 2007; Aluru et al., 2009; Page et al., 2016). Despite decades of research, the biogenic retrograde signalling pathway is still very poorly understood. What we do know has mostly come from an innovative screen by the group of Joanne Chory in which *genomes uncoupled* (*gun*) mutants were identified that retained nuclear gene expression of chloroplast-related genes after NF treatment (Susek et al., 1993). This screen now defines the *gun* phenotype: increased expression, compared to wild-type (WT), of nuclear genes following chloroplast damage. In total six original *gun* mutants have been described. GUN1 is a pentatricopeptide repeat protein with a still unknown function (Koussevitzky et al, 2007). The other GUNs are all related to the tetrapyrrole pathway (Mochizuki et al, 2001; Larkin et al, 2004; Woodson et al., 2011). Further analysis of these mutants has supported the idea that tetrapyrroles are important for plastid signalling (Vinti et al., 2000; Strand et al., 2003; Moulin et al., 2008; Mochizuki et al., 2008; Voigt et al., 2010) and our current understanding is that the synthesis of heme by ferrochelatase 1 results in a positive signal that promotes expression of nuclear-encoded chloroplast genes (Woodson et al., 2011; Terry and Smith, 2013).

Additional mutants identified through screens for a *gun* phenotype are the blue-light photoreceptor mutant *cry1* (Ruckle et al., 2007) and the *coe1* mutant lacking a functional mitochondrial transcription termination factor 4 (Sun et al., 2015). A number of *happy on norflurazon* (*hon*) mutants were also identified by screening seedlings grown on NF under lower light intensities (Saini et al., 2011). This identified one *hon* mutation in the ClpR4 subunit of the chloroplast-localized Clp protease complex (Saini et al., 2011). Other mutants with a *gun* phenotype have been identified *via* informed approaches to test potential signalling components. These include the transcription factor mutants *abi4* (Koussevitzky et al, 2007), *hy5* (Ruckle et al., 2007) and *glk1glk2* (Waters et al., 2009). Interestingly, *GOLDEN2-LIKE* (*GLK*) overexpressing plants (Leister and Kleine, 2016) have also been reported to show *gun* phenotypes, perhaps reflecting the complex relationship between the

anterograde signals by which the nucleus controls chloroplast development and retrograde signalling (Martin et al., 2016).

In 2011, Sun et al identified a PHD transcription factor associated with the chloroplast envelope, called PTM, which they proposed mediates chloroplast signals to the nucleus through cleavage in response to changes in plastid status. Accumulation of the N-terminus of the protein in the nucleus would then inhibit nuclear gene expression. Consistent with this, they reported that the *ptm* mutant has a *gun* phenotype with elevated expression compared to WT of *Lhcb* on both NF and Lin. This was a significant result for the field as it defined a mechanism for plastid signalling, and is unsurprisingly included in numerous models for this pathway (*e.g*. Chan et al, 2016; Bobik and Burch-Smith, 2015; Terry and Smith, 2013; Barajas-López et al, 2013). Subsequent studies from the same group have suggested that PTM functions in retrograde signalling from the chloroplast to regulate flowering under high light (Feng et al, 2016) and in the integration of light and chloroplast retrograde signalling during de-etiolation (Xu et al, 2016). However, the demonstration that PTM shows a *gun* phenotype and is involved in retrograde signalling has yet to be supported by additional experimental data from other groups.

Given the potential importance of PTM for our understanding of plastid signalling we have further examined the role of PTM in responses to NF and Lin in two different laboratories. For the experiments at Southampton, it was necessary for us to isolate the same insertional *ptm* mutant allele described in Sun et al (2011) from the SALK collection because this was no longer available from the authors. Isolation of the *ptm* mutant for this study, which we name here as *ptm-1*, is described in Figure S1. Analysis of gene expression after NF treatment was then performed. As shown in Figure 1A, 5 µM NF treatment using the experimental conditions (1% sucrose, 25 $\mu mol.m^{-2}.s^{-1}$ white light (WL) for 7 d) of Woodson et al (2011) resulted in no change in gene expression for a suite of five photosynthesis-related genes (including *LHCB2.1* used by Sun et al (2011) for their real-time PCR experiments) in *ptm-1* compared to WT seedlings, whereas there was clear rescue of gene expression in the control *gun5* and *gun6* mutants. Next we repeated the experiment under identical conditions (2% sucrose, 4d dark followed by 3d 120 $\mu mol.m^{-2}.s^{-1}$ WL) to those reported in Sun et al (2011). Under these conditions we also saw rescue of gene expression in *gun5* and *gun6*, but not in *ptm-1* (Figure 1B). These studies were performed using *ADF2* as a reference gene. To confirm that the lack of a *gun* phenotype in *ptm1* was not related to the choice of reference gene, we also normalised the data using *YLS8*, which gave essentially identical results (Figure S2). Finally, we examined expression under conditions we have

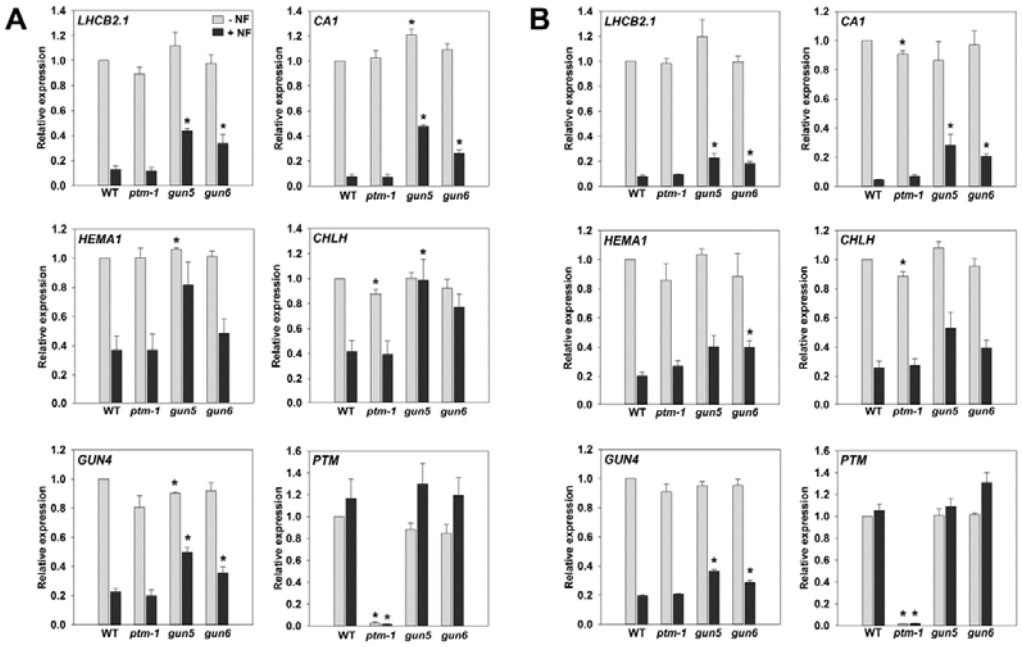

Figure 1. The *ptm-1* mutant does not show a *gun* phenotype on Norflurazon (NF). Seedlings were grown on half-strength Linsmaier and Skoog medium (A) supplemented with 1% sucrose and 0.8% agar (pH 5.7) with (dark grey bars) or without (light grey bars) 5 µM NF under continuous low white light (LWLc, 25 µmol m^{-2} s^{-1}) for 7 d, or (B) supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF under the following conditions: an initial 2 h WL treatment (120 µmol m^{-2} s^{-1}) to stimulate germination, 4 d dark, 3 d WLc (120 µmol m^{-2} s^{-1}). For (A) and (B), *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

previously described (McCormac and Terry, 2004). With 3d dark followed by 3d 120 µmol.m^{-2}.s^{-1} WL we also saw no *gun* phenotype for *ptm-1* either in the presence or absence of sucrose (Figure S3). Only under one particular set of conditions did we see any indication of a rescue of gene expression in *ptm-1* after NF treatment. Under these conditions (1% sucrose, 2d dark followed by 3d 100 µmol.m^{-2}.s^{-1} WL with a lower NF concentration of 1 µM) we saw a very small, but statistically significant increase for *LHCB2.1* and *HEMA1*, but not for the other three genes tested (Figure S4). Given that under these conditions *gun1-1* rescue was complete for both genes (>300% for *HEMA1*) we do not believe this one exception supports a role for PTM in the plastid signalling pathway exposed by NF treatment.

The *ptm-1* mutant was also reported to result in elevated gene expression compared to WT seedlings when grown on Lin (Sun et al., 2011). We therefore also tested *ptm-1* under these conditions. As shown in Figure 2, *ptm-1* failed to result in elevated gene expression on Lin while *gun1-1* (Koussevitzky et al., 2007) and *gun1-103* (see methods) control seedlings, both showed strong rescue of gene expression (Figure 2). This was true whether seedlings were grown in the dark (Figure 2A) or in the light (Figure 2B), and was independent of the reference gene used (Figure S5).

To verify further whether we could detect a *gun* mutant phenotype for *ptm* mutants, we also performed experiments in parallel in Kyoto. For this set of experiments

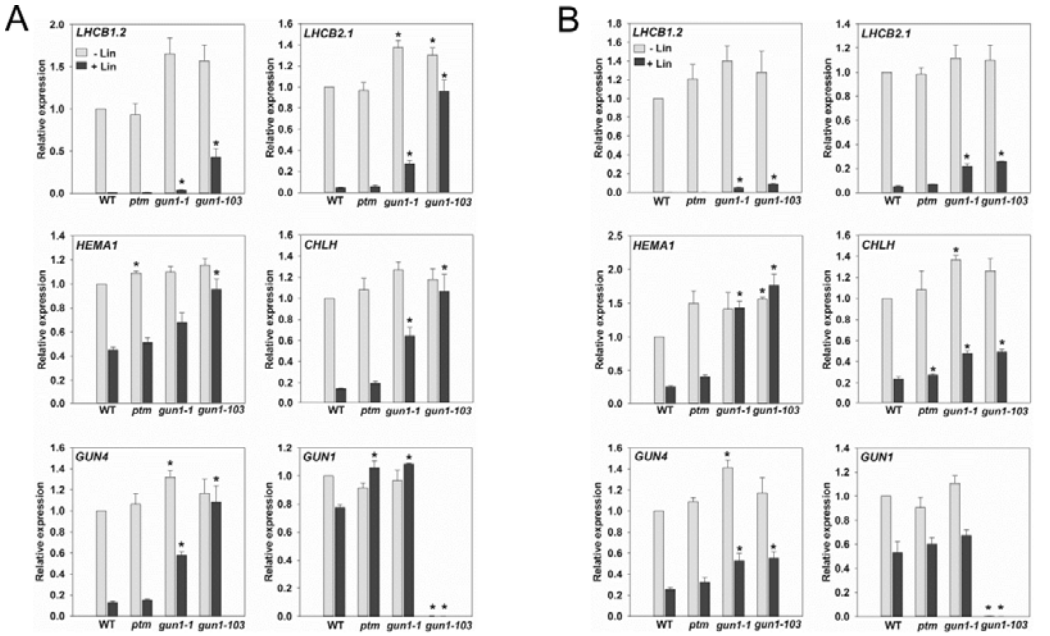

Figure 2. The *ptm* mutant does not show a *gun* phenotype on lincomycin (Lin). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin in dark for 5 d (A), or (B) on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin under the following conditions: 2 d dark, 3 d Wlc (100 µmol $m^{-2}s^{-1}$). For (A) and (B), two alleles of *genomes uncoupled 1* (*gun1-1 and gun1-103*) mutants were included as positive control (known to rescue gene expression on Lin). Expression is relative to WT -Lin and normalised to *ACTIN2 (ACT2,* At3g18780*)* used in Sun et al (2011). Data shown are means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs.* WT for the same treatment (-Lin or + Lin), Student's *t*-test ($p<0.05$).

two *ptm* alleles were used, the original *ptm* mutant (*ptm-1* OL) was obtained from Lixin Zhang (CAS, Beijing; Sun et al., 2011) and independently from the SALK collection (*ptm-1*) and, in addition, a second *ptm* allele, *ptm-2*, was also identified from the SALK collection (Fig S1). As shown in Figure 3 none of the *ptm* mutants showed an elevation of *LHCB1.2* (although the primer set used is also likely to detect *LHCB1.1* and *LHCB1.3)* or *LHCB2.1* expression after NF or Lin treatment compared to WT, while a strong increase was observed in the *gun1-1* control.

In conclusion, rigorous testing of the phenotype of *ptm* mutants on NF and Lin shows that the *ptm* mutant does not show elevated expression of photosynthetic genes compared to WT. This was true whether using the conditions described in the original publication or other conditions used routinely to test plastid signalling responses. One possible difference between our study and that of Sun et al (2011) is that they used RNA gel blot analysis for most of their experiments. The probe used should preferentially detect *LHCB1.1*, but might also be expected to detect *LHCB1.2* and *LHCB1.3,* and possibly other *LHCB* genes. In our experiments we have tested both *LHCB1.1* and *LHCB1.2,* so it remains possible that changes in another *LHCB* gene could account for the observed phenotype in the original paper (Sun et al., 2011). However, Sun et al (2011) also reported the same gene expression phenotype for *ptm* using real-time PCR and a primer pair that most closely matches *LHCB2.1,* and we did not detect an increase in expression for this gene in our

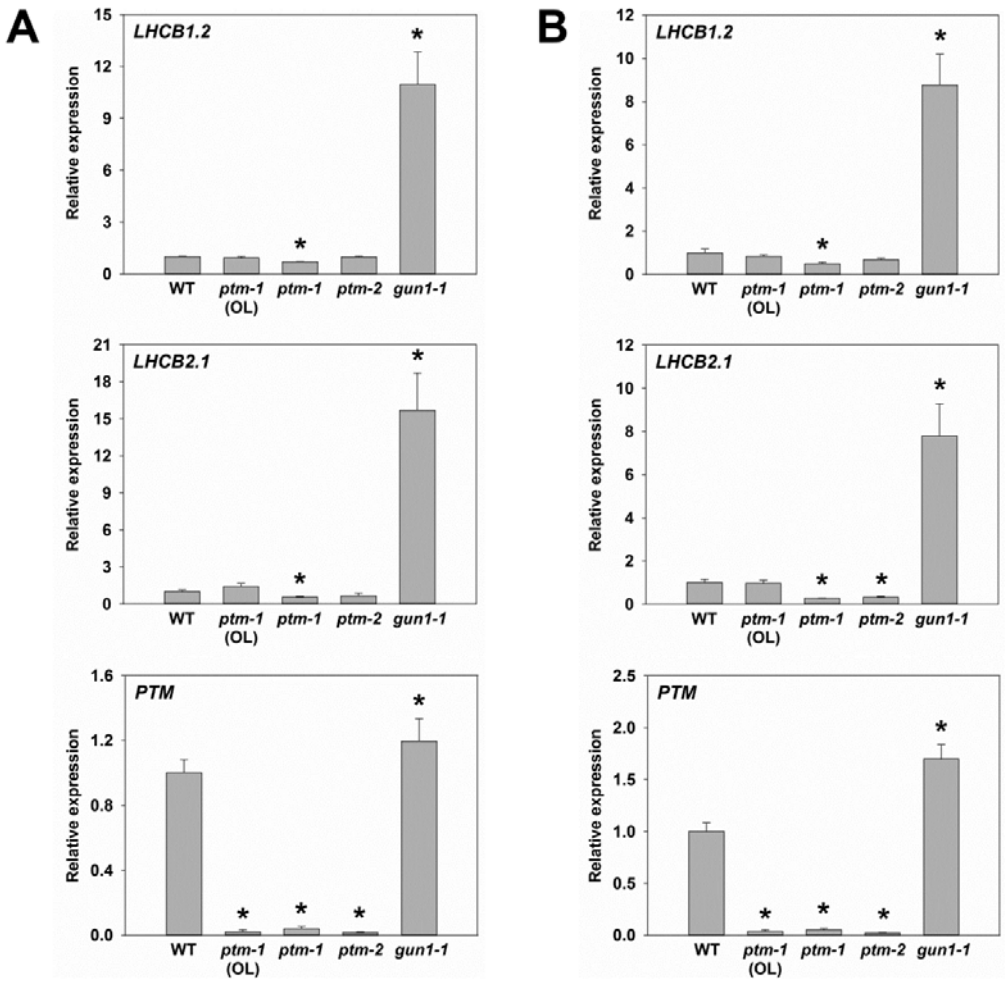

Figure 3. A second *ptm* mutant allele does not show a *gun* phenotype on Norflurazon (NF) or lincomycin (Lin). Seedlings were grown on Murashige and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8), and either (A) 2.5 µM NF or (B) 560 µM Lin. All seedlings were grown under continuous white light (WLc, 100 µmol m^{-2} s^{-1}) for 4 d at 23 °C. Three *ptm* mutant lines were tested: *ptm-1* (OL) is the original line as used in Sun *et al*., 2011; *ptm-1* is the same insertion line as *ptm-1* (OL), Salk_013123, but obtained independently from the stock centre; *ptm-2* is a second insertion line, Salk_073799. The *genomes uncoupled 1-1* (*gun1-1*) mutant was included as a positive control (known to rescue nuclear gene expression on NF and Lin). Expression was determined with qRT-PCR and is relative to WT +NF and normalised to *TUBULIN BETA CHAIN 2* (*TUB2*, At5g62690). Data shown are the means +SEM of five independent biological replicates. Asterisks denote a significant difference *vs*. WT +NF, Student's *t*-test ($p<0.05$).

experiments (with one exception). We therefore believe it is unlikely that differences in detection methods or genes tested can account for the observed differences in phenotype. Moreover, if PTM is to be considered an important player in plastid signalling, the *gun* phenotype of *ptm* should be robust enough to withstand this level of scrutiny. We have not tested other results reported by Sun et al (2011). However, we note that the 3-fold elevation of expression of *PTM* on NF measured using *PTM:GUS* was not apparent in our experiments (Figures 1 and S3) and the reduction in *PTM* expression in *gun1* after NF and Lin treatment was also not observed (Figure 3). In fact *PTM* expression was moderately (but significantly)

elevated in *gun1-1* in our study (Figure 3). Whether our result has implications for other PTM signalling roles (Feng et al., 2016; Xu et al., 2016) is currently unknown, but should be the subject of further scrutiny.

The signalling pathway by which the status of the developing chloroplast is relayed to the nucleus is one of the few remaining plant signalling pathways that we know of, but for which we have little idea of the signalling components involved. We believe this study resolves one of the major discrepancies in plastid signalling research by eliminating a major role for PTM, and paves the way for more focussed studies that build on recent progress on the role of tetrapyrroles and chloroplast protein homeostasis in plastid retrograde signalling (Woodson et al., 2011; Murata et al., 2015; Ibata et al., 2016; Tadini et al., 2016).

Supplemental data

Supplemental Table. Primers used in this study.

Supplemental Figure S1. Characterisation of the *ptm* T-DNA insertion mutants

Supplemental Figure S2. The phenotype of *ptm-1* after NF treatment using the Sun et al (2011) method normalised to *YLS8*

Supplemental Figure S3. The phenotype of *ptm-1* after NF treatment using the McCormac & Terry (2004) method in the presence and absence of sucrose

Supplemental Figure S4. The phenotype of *ptm-1* after NF treatment using a modification of the McCormac & Terry (2004) method in the presence of sucrose

Supplemental Figure S5. The phenotype of *ptm-1* after Lin treatment normalised to *YLS8*

Acknowledgments

We would like to thank Tania Garcia-Becerra for technical support. Thanks also to Joanne Chory and Jesse Woodson (SALK Institute) for the *gun1-1*, *gun5* and *gun6* mutants used in this study. N.M. thanks Lixin Zhang (Chinese Academy of Sciences) for the *ptm* (*ptm-1* OL) mutant.

Figure Legends

Figure 1. **The *ptm-1* mutant does not show a *gun* phenotype on Norflurazon (NF).**
Seedlings were grown on half-strength Linsmaier and Skoog medium (A) supplemented with

1% sucrose and 0.8% agar (pH 5.7) with (dark grey bars) or without (light grey bars) 5 µM NF under continuous low white light (25 µmol.m^{-2}.s^{-1}) for 7 d, or (B) supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF under the following conditions: an initial 2 h WL treatment (120 µmol.m^{-2}.s^{-1}) to stimulate germination, 4 d dark, 3 d WLc (120 µmol m^{-2} s^{-1}). For (A) and (B), *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

Figure 2. The *ptm* mutant does not show a *gun* phenotype on lincomycin (Lin). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin in dark for 5 d (A), or (B) on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin under the following conditions: 2 d dark, 3 d WL (100 µmol.m^{-2}.s^{-1}). For (A) and (B), the *genomes uncoupled, gun1-1* and *gun1-103* mutants were included as positive controls (known to rescue gene expression on Lin). Expression is relative to WT -Lin and normalised to *ACTIN2* (*ACT2*, At3g18780) used in Sun et al. (2011). Data shown are means +SEM of three independent biological replicates. Asterisks denote a significant difference vs. WT for the same treatment (-Lin or + Lin), Student's t-test ($p<0.05$).

Figure 3. **A second *ptm* mutant allele does not show a *gun* phenotype on Norflurazon (NF) or lincomycin (Lin).** Seedlings were grown on Murashige and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8), and either (A) 2.5 µM NF or (B) 560 µM Lin. All seedlings were grown under continuous white light (WLc, 100 µmol.m^{-2}.s^{-1}) for 4 d at 23 °C. Three *ptm* mutant lines were tested: *ptm-1* (OL) is the original line as used in Sun *et al*., 2011; *ptm-1* is the same insertion line as *ptm-1* (OL), Salk_013123, but obtained independently from the stock centre; *ptm-2* is a second insertion line, Salk_073799. The *genomes uncoupled 1-1* (*gun1-1*) mutant was included as a positive control (known to rescue nuclear gene expression on NF and Lin). Expression was determined with qRT-PCR and is relative to WT +NF and normalised to *TUBULIN BETA CHAIN 2* (*TUB2*, At5g62690). Data shown are the

means +SEM of five independent biological replicates. Asterisks denote a significant difference *vs*. WT +NF, Student's *t*-test ($p<0.05$).

Supplemental Fig. S1. **Characterisation of the *ptm* T-DNA insertion mutants.** (A) *PTM* gene structure, with black boxes representing exons. The approximate location of the Salk_013123 (*ptm-1*) and Salk_073799 (*ptm-2*) T-DNA inserts, genotyping primers (LB, LP, RP) and qRT-PCR primers (qF1, qR1, qF2, qR2) are indicated. The *ptm-2* mutant has tandem T-DNA insertions with a 24 bp deletion, in which the LB primer binding site is located at each end of the tandem insertion. Precise T-DNA insertion sites in (B) *ptm-1* and (C) *ptm-2* as revealed by sequencing. For (B) and (C) amino acid single letter codes are given above DNA sequences, with the T-DNA sequences underlined in black. Sequence is given from the LP and RP sides of the *ptm-2* T-DNA insertion in (C), to demonstrate the site of the 24 bp deletion (underlined in red in the WT sequence). (D) PCR genotyping of *ptm-1* and *ptm-2* mutants. Primers shown in (A) were used to amplify the following: *ptm-1* - WT band (LP1 + RP1, predicted size 1,098 bp) and mutant band (LB + RP1, predicted size 687 bp); *ptm-2* - WT band (LP2 + RP2, predicted size 1,142 bp) and two mutant bands (LB + RP2, predicted size 661 bp, and LB + LP2, predicted size 904 bp). MW = molecular weight marker. (E) Expression of *PTM* in WT and *ptm-1* seedlings as determined by qRT-PCR. This analysis was repeated under the conditions used in this study: the growth conditions in McCormac & Terry, 2004 (white bars), Sun *et al*., 2011 (grey bars) and Woodson *et al*., 2011 (black bars), all in the absence of NF. Expression is relative to WT for each condition and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data represent the mean + SEM of three independent biological replicates, asterisks indicate a significant difference *vs*. WT ($p < 0.05$, Student's *t*-test).

Supplemental Fig. S2. **Normalisation of expression data to a different reference gene does not reveal a *gun* phenotype for *ptm-1*.** Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF under the following conditions: an initial 2 h WL treatment (120 $\mu mol.m^{-2}.s^{-1}$) to stimulate germination, 4 d dark, 3 d WLc (120 $\mu mol\ m^{-2}\ s^{-1}$). *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *YELLOW LEAF SPECIFIC GENE 8* (*YLS8*, At5g08290). Data shown are the means +SEM of three independent biological replicates. Asterisks

denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

Supplemental Fig. S3. **Growth under a third set of conditions fails to find a *gun* phenotype in *ptm-1*.** Seedlings were sown onto half-strength Murashige and Skoog medium supplemented with 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF, and either in the presence (A) or absence (B) of 1% sucrose. For (A) and (B), seedlings were grown under the following conditions: an initial 2 h WL treatment (120 $\mu mol.m^{-2}.s^{-1}$) to stimulate germination, 3 d dark, 3 d WLc (120 $\mu mol.m^{-2}.s^{-1}$). *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

Supplemental Figure S4. The *ptm* mutant shows a very weak *gun* phenotype for some genes under low (1 µM) Norflurazon (NF). Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 1 µM NF under the following conditions: 2 d dark, 3 d WLc (100 $\mu mol.m^{-2}.s^{-1}$). The *genomes uncoupled 1* (*gun1-1*) mutant was included as positive control (known to rescue gene expression on NF). Expression is relative to WT -NF and normalised to *YELLOW LEAF SPECIFIC GENE 8 (YLS8,* At5g08290). Data shown are means +SEM of three independent biological replicates. Asterisks denote a significant difference vs. WT for the same treatment (-NF or + NF), Student's t-test ($p<0.05$).

Supplemental Figure S5. The *ptm* mutant does not show a *gun* phenotype on lincomycin (Lin). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin in dark for 5 d (A), or (B) on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM LIN under the following conditions: 2 d dark, 3 d Wlc (100 $\mu mol.m^{-2}.s^{-1}$). For (A) and (B), two alleles of *genomes uncoupled 1* (*gun1-1* and *gun1-103*) mutants were included as positive control (known to rescue gene expression on Lin). Expression is relative

284 to WT -Lin and normalised to *YELLOW LEAF SPECIFIC GENE 8 (YLS8,* At5g08290). Data shown
285 are means +SEM of three independent biological replicates. Asterisks denote a significant
286 difference vs. WT for the same treatment (-Lin or + Lin), Student's t-test ($p<0.05$).
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